Anal. Chem. **2007,** *79,* 4840−4844

Profiling of Organic Acids during Fermentation by Ultraperformance Liquid Chromatography−Tandem Mass Spectrometry

Keri Lyn Ross, Travis T. Tu, Sean Smith, and Joseph J. Dalluge*

Cargill Global Food Technology Group, Cargill Incorporated, P.O. Box 5702, Minneapolis, Minnesota 55440-5702

A method has been developed for rapid quantification of organic acids using ultraperformance liquid chromatography/electrospray−tandem mass spectrometry (UPLC/ESI-MS−MS) to monitor the metabolism of 10 organic acids during microbial fermentation. Because comprehensive chromatographic separation is not required, analysis time is less than traditional ion chromatography assays, with complete organic acid analyses by UPLC/ESI-MS−MS being achieved in less than 3 min. Quantification is accomplished using nine isotopically labeled organic acids as internal standards. Intrasample precisions for organic acid measurements in fermentation supernatants using this method average 8.9% (RSD). Calibration curves are linear over the range of 0.06−100 μg/mL, and detection limits are estimated at 0.06−1 μg/mL. This method has the potential to demonstrate correlation of organic acid consumption and production by microorganisms with observed growth profiles, novel media formulations, and cellular growth events. Data visualization software has been used to profile organic acid levels during fermentation and correlate these profiles to nutrient supplementation protocols employed during microbial production. The potential use of this capability in computational modeling and simulation of microbial metabolism to accelerate the bioprocess development cycle is recognized.

Metabolic engineering and bioprocess optimization are playing increasingly significant roles in the current pursuit of sustainable economic systems based on the microbial production of value-added chemicals from renewable plant biomass.[1−3] As directed strain improvement by metabolic engineering is based on a detailed knowledge of metabolic pathways and their regulation, the development of rapid and effective analytical methods for the measurement of key intracellular and extracellular metabolites becomes paramount. Data compiled from such methods can be employed in modeling and optimization of production processes, monitoring activity of metabolic pathways, and determining the ability of the metabolites of interest to augment or inhibit microbial growth and production. Because organic acids are important metabolites in fermentation, methods capable of rapid measurement of organic acid levels during fermentation have significant potential from the standpoint of strain selection and improvement, optimization of media formulations, profiling microbial metabolism and growth state, and functional genomics approaches to computational modeling and simulation of microbial metabolism.[3]

Methods of choice for the measurement of organic acids have traditionally been based on liquid chromatography (LC) combined with refractive index (RI),[4,5] conductivity,[6] or ultraviolet (UV) detection.[6,7] These methods are time-consuming and limited generally by a narrow linear dynamic range (1 order of magnitude), high limits of detection (g/L), and susceptibility to matrix interferences.[4−7]

This article describes the development of a method based on UPLC/ESI-MS−MS for the rapid quantification (less than 3 min per analysis) of organic acids to monitor the metabolism of 10 organic acids during microbial fermentation. The application of this method to the determination of organic acids in cell extracts has also been demonstrated. Data visualization software has been used to profile organic acid levels during fermentation and correlate the resulting profiles to nutrient supplementation protocols employed during microbial production. The potential of this method as an aid to computational modeling and simulation of microbial metabolism to accelerate the bioprocess development cycle is discussed.

EXPERIMENTAL SECTION

Reagents. OmniSolv HPLC-grade acetonitrile (ACN) was obtained from EMD Chemicals Inc. (Darmstadt, Germany). Optima ultrapure water was obtained from Thermo Fisher Scientific Inc. (Waltham, MA). Organic acids were purchased from Sigma Chemical Co. (St. Louis, MO). Deuterated internal standards $^{2}H_3$-succinic, $^{2}H_4$-citric, $^{2}H_3$-malic, $^{2}H_3$-acetic, and $^{2}H_3$-propionic acids were obtained from CDN Isotopes (Quebec, Canada). ^{13}C-labeled internal standards $^{13}C_3$-malonic, $^{13}C_3$-lactic, and $^{13}C_3$-pyruvic acids were purchased from Cambridge Isotope Laboratories (Andover, MA). $^{13}C_3$-3-Hydroxypropionic (3-HP) acid was

* Author to whom correspondence should be addressed. Phone: 952-742-3038. Fax: 952-742-3010. E-mail: joseph_dalluge@cargill.com.

(1) Wendisch, V. F.; Bott, M.; Eikmanns, B. J. *Curr. Opin. Microbiol.* **2006**, *9*, 268−274.
(2) van der Werf, M. J. *Trends Biotechnol.* **2005**, *23*, 11−16.
(3) Mahadevan, R.; Burgard, A. P.; Famili, I.; Van Dien, S.; Schilling, C. H. *Biotechnol. Bioprocess Eng.* **2005**, *10*, 408−417.
(4) Weigang, F.; Reiter, M.; Jungbauer, A.; Katinger, H. *J. Chromatogr.* **1989**, *497*, 59−68.
(5) Calull, M.; Marce, R. M.; Borrull, F. *J. Chromatogr.* **1992**, *590*, 215−222.
(6) Widiastuti, R.; Haddad, P. R.; Jackson, P. E. *J. Chromatogr.* **1992**, *602*, 43−50.
(7) Rajakyla, E. *J. Chromatogr.* **1981**, *218*, 695−701.

10.1021/ac0624243 CCC: $37.00
Published on Web 05/26/2007

Table 1. Selected Ions or MRM Transitions Monitored and Internal Standards Employed for the Measurement of 10 Organic Acids

organic acid	precursor [M − H]⁻	fragment [M − H]⁻ [a]	internal standard	average RSD (%) [b]
acetic acid	59		$^{2}H_3$-acetic acid	13.3
$^{2}H_3$-acetic acid	62			
propionic acid	73		$^{2}H_3$-propionic acid	10.7
$^{2}H_3$-propionic acid	76			
pyruvic acid	87		$^{2}H_3$-pyruvic acid	11.1
$^{2}H_3$-pyruvic acid	90			
lactic acid	89	43	$^{13}C_3$-lactic acid	8.5
3-HP	89	59	$^{13}C_3$-3HP	
$^{13}C_3$-lactic acid	92	45		
$^{13}C_3$-3HP	92	61		
malonic acid	103	59	$^{13}C_3$-malonic acid	
$^{13}C_3$-malonic acid	106	61		
fumaric acid	115	71	$^{2}H_3$-succinic acid [c]	6.5
succinic acid	117	73	$^{2}H_3$-succinic acid	8.0
$^{2}H_3$-succinic acid	121	77		
malic acid	133	115	$^{2}H_3$-malic acid	5.1
$^{2}H_3$-malic acid	136	117		
citric acid	191	111	$^{2}H_4$-citric acid	7.1
$^{2}H_4$-citric acid	195	113		

[a] Fragment ions are not listed for acids monitored by SIR. [b] Relative standard deviations were calculated for fermentation supernatant samples and are only listed for those organic acids that were detected in fermentations 1−4 profiled in this study. [c] The available labeled form of fumaric acid, $^{2}H_2$-fumaric acid, and succinic acid have identical mass spectral fragmentation behavior and are not separated in the method described herein. Therefore, $^{2}H_3$-succinic acid is used as an internal standard for the measurement of fumaric acid.

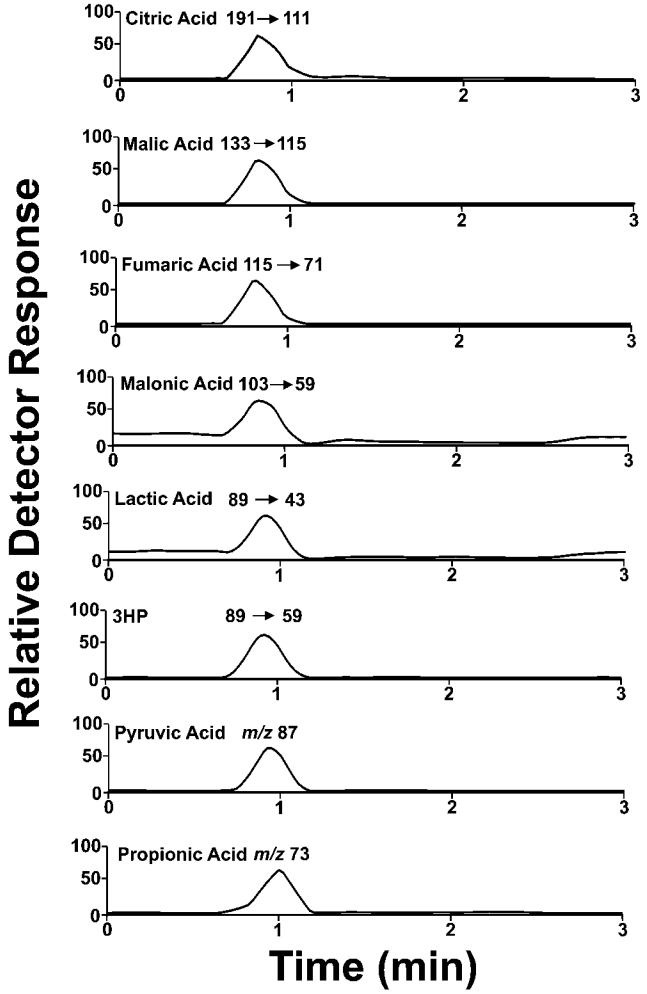

Figure 1. UPLC/ESI-MS−MS determination of organic acids during fermentation. Individual MRM or SIR (pyruvic acid and propionic acid) transitions for eight selected organic acids are illustrated.

produced in-house via enzymatic reaction. Difco LD yeast extract was obtained from Becton Dickinson, Inc. (Sparks, MD). NH_4Cl was purchased from VWR Inc. (West Chester, PA). KH_2PO_4, $MnSO_4{\cdot}H_2O$, $FeSO_4{\cdot}7H_2O$, and $MgSO_4{\cdot}7H_2O$ were obtained from J.T. Baker Co. (Phillipsburg, NJ). L-Threonine, L-isoleucine, L-serine, L-glycine, pyridoxal, vitamin B12, glucose, kanamycin, and IPTG were purchased from Sigma Chemical Co. (St. Louis, MO).

Directly Combined Ultraperformance Liquid Chromatography/Electrospray Ionization−Tandem Mass Spectrometry. The system used for separation, identification, and quantification of organic acids consisted of a Waters Acquity UPLC coupled to a Waters Quattro Premier XE triple-quadrupole mass spectrometer. Separation was achieved with two Thermo Fisher Scientific Inc. (Waltham, MA) Hypersil Gold (50 mm × 2.1 mm, 1.9 μm particles) columns in series at 30 °C. An isocratic flow of 97% water and 3% ACN at a flow rate of 0.3 mL/min was used. Electrospray ionization tandem mass spectrometric methods (ESI-MS−MS) were created for seven compounds (lactic acid, 3-HP, malonic acid, fumaric acid, succinic acid, malic acid, citric acid) using unique multiple reaction monitoring (MRM) transitions optimized by direct infusion of individual organic acids. Selected ion recording (SIR) of three additional organic acids (acetic, propionic, pyruvic) allows simultaneous quantification of 10 acids in complex biological matrices employing 9 isotopically labeled organic acids as internal standards. Parameters of the ESI-MS−MS system were selected based on in-source generation of the deprotonated molecular ions of each organic acid as well as production of compound-specific fragment ions. The following parameters were used for ESI-MS−MS analysis of the 19 organic acids in negative ionization mode: capillary, 3.25 kV; cone, 20 V; extractor, 3 V; rf lens, 0.2 V; source temperature, 100 °C; desolvation temperature, 350 °C; cone gas flow, 47 L/h; low-mass resolution (Q1), 15 V; high-mass resolution (Q1), 15 V; ion energy (Q1), 0.5 V; entrance, −5 V; exit, 1 V; low-mass resolution (Q2), 15 V; high-mass resolution (Q2), 15 V; ion energy (Q2), 1.0 V; multiplier 650 V. Collision energies were varied to effect optimal fragmentation of each analyte and were set as follows: 6 V for acetic, $^{2}H_3$-acetic, propionic, $^{2}H_3$-propionic, pyruvic, and $^{2}H_3$-pyruvic acids; 11 V for lactic, $^{13}C_3$-lactic, 3-HP, $^{13}C_3$-3-HP, and fumaric acids; 13 V for malonic, $^{13}C_3$-malonic, succinic, $^{2}H_3$-succinic, malic, $^{2}H_3$-malic, citric, and $^{2}H_4$-citric acids. Multiple reaction monitoring parameters were as follows: interscan delay, 0.05 s; interchannel delay, 0.05 s; dwell time, 0.03 s.

Microbial Growth Conditions. *Escherichia coli* (K12) fermentations were carried out under aerobic conditions (>40% O_2) in 5 L fed-batch fermentors at pH 6.5 and 31 °C. Growth media for all fermentations was comprised of 4 g/L yeast extract, 6.5 g/L NH_4Cl, 2 g/L $(NH_4)_2SO_4$, 3 g/L KH_2PO_4, 1 g/L L-threonine, 1 g/L L-isoleucine, 30 mg/L $MnSO_4{\cdot}H_2O$, 10 mg/L $FeSO_4{\cdot}7H_2O$, 1 g/L $MgSO_4{\cdot}7H_2O$, 2 mg/L pyridoxal (B6), 2 mg/L vitamin B12, 40 g/L glucose, 50 mg/L kanamycin, 0.4 mL of 1 M IPTG, and 4% inoculum. When glucose levels fell below 2 g/L, the fermentations were supplemented with 28 g/L $(NH_4)_2SO_4$, 13.25 g/L

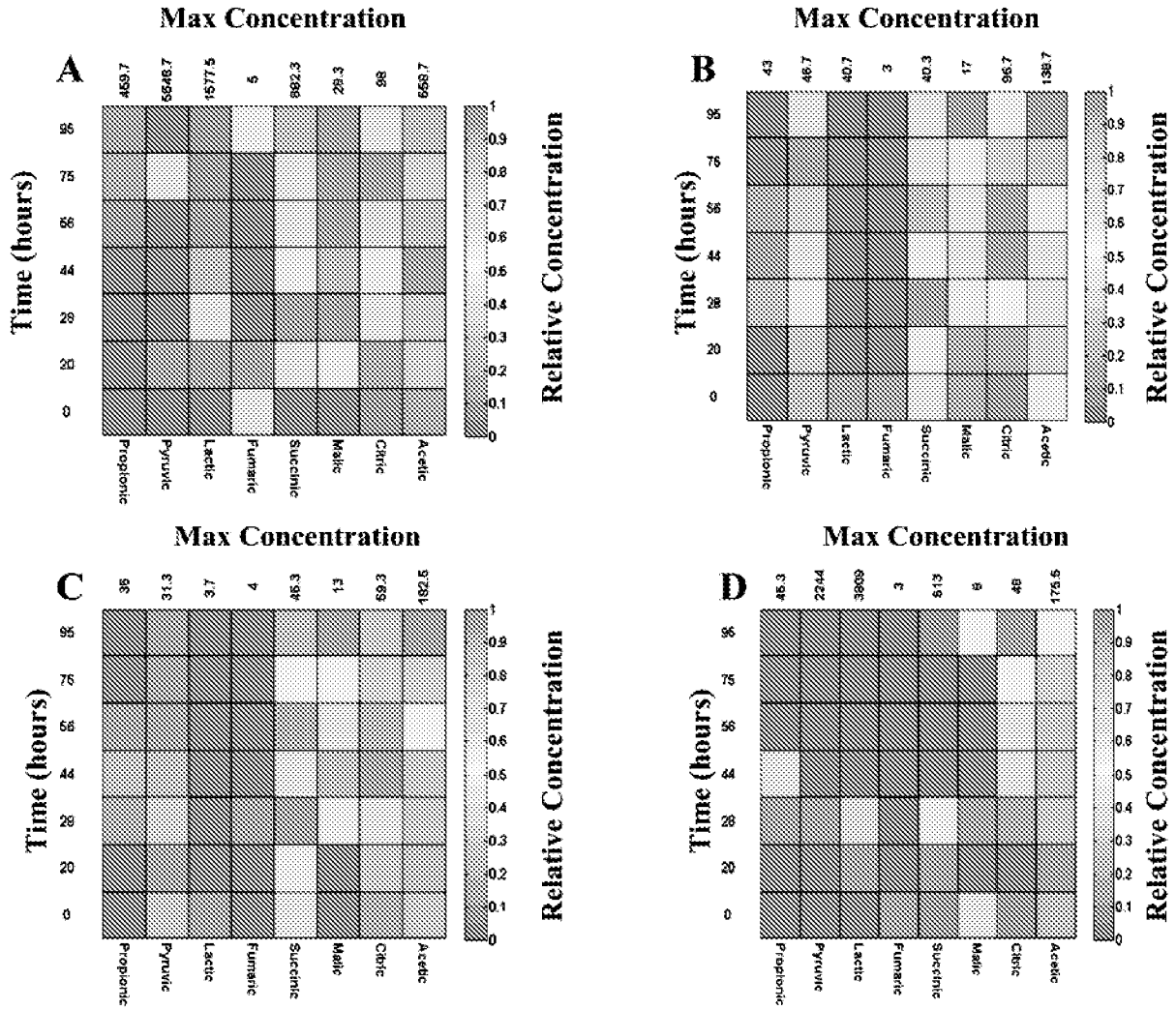

Figure 2. Organic acid profiles for *E. coli* fermentations employing different nutrient supplementation protocols. (A) Fermentation 1: control. (B) Fermentation 2: supplementation with serine. (C) Fermentation 3: supplementation with glycine. (D) Fermentation 4: supplementation with serine plus inorganic nitrogen. Maximum concentrations are in units of μg/mL (ppm).

NH_4Cl (fermentations 1–3) or 34.7 g/L NH_4Cl (fermentation 4), 1.5 g/L L-threonine, 4 g/L yeast extract, 3 g/L KH_2PO_4, 1 mg/L vitamin B12, 400 g/L glucose, 10 g/L L-serine (fermentations 2 and 4 only), and 10 g/L L-glycine (fermentation 3 only).

Sample Preparation and Analysis. For standardization, four levels of calibration mixtures were prepared for the 10 organic acids and 9 internal standards (Table 1) to achieve four different mass ratios for each of the organic acids in the mixtures. These solutions were then analyzed by UPLC/ESI-MS–MS, and the data were subjected to a linear least-squares analysis.

The method involved addition of precisely known amounts of nine isotopically labeled organic acid internal standards to a 10:1 dilution of filtered (0.2 μm) fermentation samples collected at 0, 20, 28, 44, 56, 75, and 95 h, followed by direct UPLC/ESI-MS–MS analysis. The peak area ratios (organic acid/corresponding IS) were then used in conjunction with the calibration curves to derive the concentration of individual organic acids in the samples.

Data Correlation and Visualization. All organic acid profile visualization and correlation data and figures were completed using Matlab v6.5 (The Mathworks, Inc., Natick, MA).[8,9]

RESULTS AND DISCUSSION

The purpose of this study was to develop a method to rapidly measure organic acid composition and concentration during fermentation using UPLC/ESI-MS–MS and apply this methodology to fermentation profiling. Development of the method was achieved by testing several LC columns, flow rates, and mobile phase compositions to effect optimal retention and separation of the organic acids and minimization of matrix interference. Columns tested included the following: Waters Acquity C_{18} BEH 50 mm × 2.1 mm (1.7 μm); Waters Acquity C_8 BEH 50 mm × 2.1 mm (1.7 μm); Waters Acquity C_{18} BEH Shield 50 mm × 2.1 mm (1.7 μm); Phenomenex Aqua C_{18} 100 mm × 2 mm (3 μm); Agilent SB-C_{18} 100 mm × 2.1 mm (1.8 μm); Agilent XDB-C_{18} 100 mm × 2.1 mm (1.8 μm); Thermo Hypersil Gold C_{18} 50 mm × 2.1 mm (1.9 μm). Acceptable retention of the organic acids was achieved only with the Thermo Hypersil Gold column

(8) Box, G. E. P.; Hunter, W. G.; Hunter, J. S. *Statistics for Experimenters: An Introduction to Design, Data Analysis and Model Building*; Wiley: New York, 1978.

(9) Dalluge, J. J.; Smith, S.; Sanchez-Riera, F.; McGuire, C.; Hobson, R. *J. Chromatogr., A* **2004**, *1043*, 3–7.

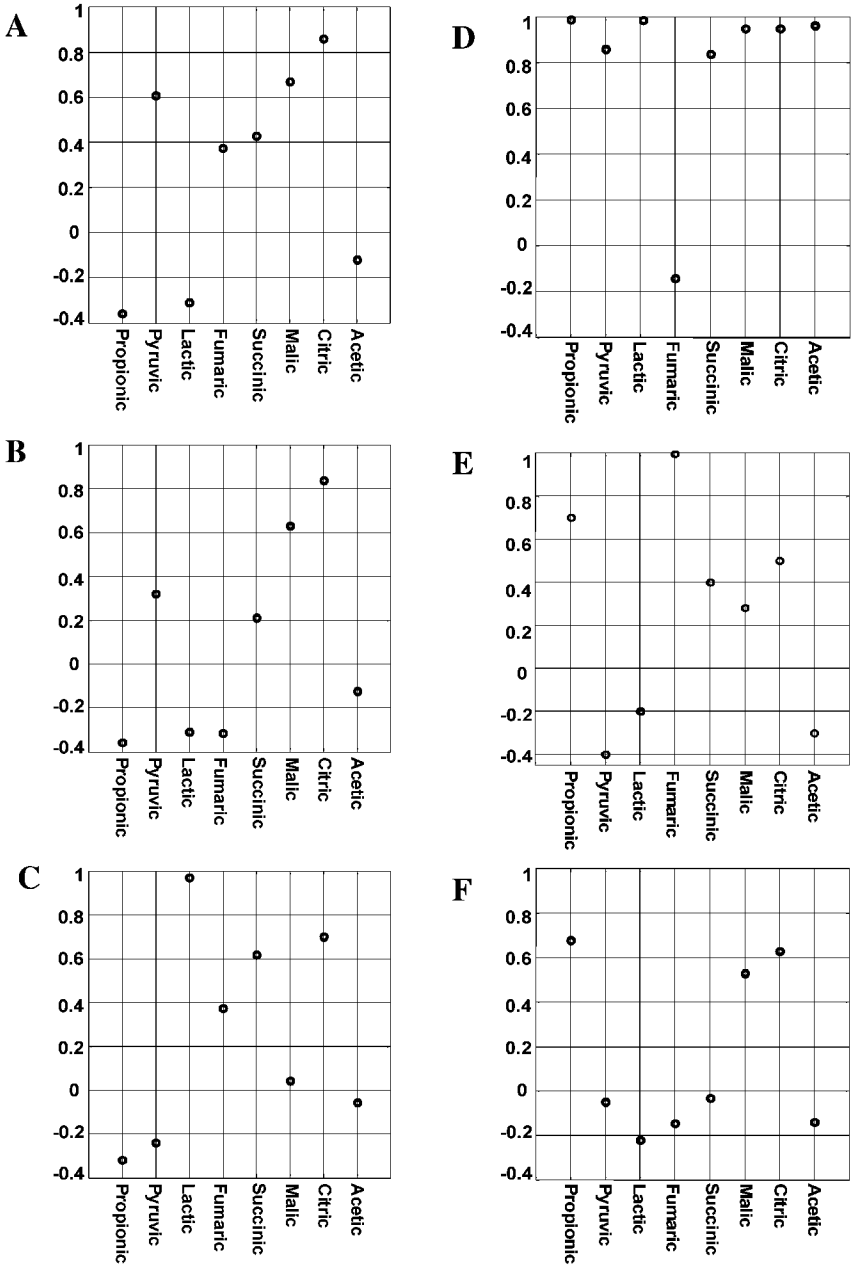

Figure 3. Correlation of individual organic acid profiles in four *E. coli* fermentations. Standard univariate correlation coefficients (R) are plotted for each of eight organic acids measured in four side-by-side fermentation runs. (A) Correlation plot of fermentations 1 and 2. (B) Correlation plot of fermentations 1 and 3. (C) Correlation plot of fermentations 1 and 4. (D) Correlation plot of fermentations 2 and 3. (E) Correlation plot of fermentations 2 and 4. (F) Correlation plot of fermentations 3 and 4.

employing an isocratic elution in 97:3 water/ACN (v/v). Interference from coeluting matrix components has not been observed, and addition of isotopically labeled organic acid internal standards compensates for any ionization suppression due to matrix effects. Calculated recoveries of organic acids in spiked matrix samples (n = 3) average 98%. The method developed here is capable of measuring a suite of 10 organic acids in less than 3 min per sample and is applicable also to the measurement of organic acids in cell extracts, rare organic acids, and structural isomers of naturally occurring organic acids (data not shown). A description of this method and its use for correlation of organic acid profiles to nutrient supplementation protocols employed during microbial production follows.

Typical MRM chromatograms for the determination of eight representative organic acids are illustrated in Figure 1. MRM transitions monitored for each organic acid and internal standard, the identities of the internal standards employed for quantification of each organic acid, and average RSD values for the measurement of each organic acid detected in the samples are tabulated in Table 1. The following advantages of leveraging the selectivity of tandem mass spectrometry for the measurement of organic acids were immediately noted: (1) chromatographic separation of each analyte is not required (cross-channel interference from coeluting organic acids has not been observed) allowing simultaneous measurement of all organic acids monitored in less than 3 min per sample; (2) tandem mass spectrometry distinguishes structural

isomers such as 3-HP and 2-hydroxypropionic acid (lactic acid) based on characteristic fragmentation behaviors of individual organic acids (Figure 1). Calibration curves were linear over the range of 0.06−100 μg/mL (average correlation coefficient 0.998, all correlation coefficients >0.996), and detection limits calculated for the organic acids were in the range of 0.06−1 μg/mL. Intrasample precisions for organic acid measurements in fermentation supernatants averaged 8.9% (average RSD of all triplicate measurements, data not shown). This preliminary statistical data suggest that the method is capable of profiling organic acid metabolism in fermentation samples.

To demonstrate the feasibility of the method described herein for generating and visualizing organic acid profiles and correlating them to growth conditions, organic acids were measured during four *E. coli* fermentations differentiated by unique nutrient supplementation protocols. Fermentation 1 represented a control fermentation, fermentation 2 was supplemented with the amino acid serine, fermentation 3 was supplemented with the amino acid glycine, and fermentation 4 was supplemented with serine as well as additional inorganic nitrogen-containing feedstock material. Organic acid profiles for each fermentation are illustrated in Figure 2. The values used to generate the profiles are the respective means of triplicate runs for each sample time point. The concentration of each organic acid over the course of the fermentation is normalized to its maximum concentration (defined as 1.0) and then mapped to a pseudocolor scale from deep blue (0) to dark brown (1). Included with each graph as a visual reference is the actual color scale. Also noted is the maximum concentration of each organic acid (at the top of the *y*-scale), allowing a semiquantitative comparison of organic acid concentrations, one with another over time, as well as the ability to follow overall trends for individual organic acids. When the data are visualized in this way, it is observed that individual profiles correlate with similarities in nutrient supplementation. For example, fermentations 2 and 3, while distinct from the control fermentation, have very similar profiles, consistent with the addition to each fermentation of an amino acid as supplemental nitrogen, while additional supplementation with inorganic nitrogen results in the unique profile corresponding to fermentation 4 (Figure 2).

To better understand and visualize the specific organic acid profiles that are characteristic of a given nutrient supplementation protocol, and identify organic acids for which profiles are independent of differing growth conditions, correlation coefficients (*R* values) were calculated for each of the measured organic acids between the six possible pairs of fermentations according to the following relation:

$$R = \frac{\sum (D_{Fx} - \mu_{Fx})(D_{Fy} - \mu_{Fy})/N_D - 1}{\sigma_{Fx}\sigma_{Fy}}$$

where D_{Fx} are the set of seven time point measurements for the first fermentation in a given pairing, D_{Fy} are the points for the second fermentation in the pairing, $N_D - 1$ is the degrees of freedom of the R calculation (6 in this case), μ is the mean of the individual organic acid concentrations measured over the seven time points, and σ is the standard deviation of these values.[8] The resulting correlation plots are illustrated in Figure 3. These analyses allow identification of correlated and anticorrelated organic acids between fermentations and an alternative means to profile and compare fermentations run under different conditions. It is observed, for example, that the correlation plot comparing fermentations 2 and 3 (Figure 3D), as one might expect based on the similarity of nutrient supplementation protocols for each, illustrates a high degree of correlation of seven of the eight organic acids measured (measured fumaric acid levels were at or below the detection limit of the method at all time points, precluding any conclusions based on the anticorrelation of this analyte between fermentations 2 and 3). By contrast, a comparison of fermentations 1 and 4 (Figure 3C) illustrates a high degree of anticorrelation of measured organic acid concentrations over time, indicative of the significantly different growth conditions present in these fermentors. It is also observed that profiles of citric acid are very consistent among the fermentations (as reflected by correlation coefficients >0.6 for all comparisons) regardless of subtle changes in nutrient supplementation, indicating that this organic acid is not a relevant indicator of the changes in growth conditions applied here. It is evident from these observations that profiles such as those illustrated in Figures 2 and 3, generated under a broad assortment of growth conditions, and in combination with profiles generated for other key cellular metabolites,[9] could have a significant impact on computational modeling approaches to simulation of microbial metabolism.[10] In addition, they would aid a more thorough understanding of key microbial growth trends and physiological events that would allow a more rational and effective approach to bioprocess development.

CONCLUSIONS

A rapid method for the simultaneous measurement of underivatized organic acids during fermentation has been developed. This method has the potential to provide valuable information for optimization of media formulations, nitrogen and carbon source selection, and the diagnosis of significant host strain physiological events. This capability should find widespread use in accelerating directed strain improvement by metabolic engineering.

Received for review December 22, 2006. Accepted April 3, 2007.

AC0624243

(10) Voesky, B.; Votruba, J. *Modeling and Optimization of Fermentation Processes*; Elsevier Science Publishers B.V.: Amsterdam, 1992.